DESCRIPTION

METHOD OF CONDUCTING HOMOLOGOUS RECOMBINATION

TECHNICAL FIELD

The present invention relates to a method of efficiently conducing homologous recombination, and homologous recombinant cells obtained by the above method.

BACKGROUND ART

To date, mainly two recombination pathways, namely, a homologous recombination pathway and a non-homologous recombination pathway have been identified in eukaryotic cells. Homologous recombination is induced by the interaction between homologous sequences of DNA, whereas non-homologous recombination is irrelevant to such DNA homology and it is considered to conduct a direct ligation of cleaved double-stranded ends. In the case of budding yeast, a homologous recombination system has mainly been used as a recombination mechanism. If foreign DNA has a portion homologous to the genomic sequence of DNA, into which it is to be incorporated, at both ends thereof, the foreign DNA can be incorporated into the genomic site homologous to the sequence (Takata et al., 1997; Wach et al., 1994). It has been reported that Rad51, Rad52, and Rad54 are essential in this process (Nickoloff and Hoekstra, 1998). On the other hand, many other living bodies including humans, plants, insects, and fission yeasts have mainly used a non-homologous recombination system as a recombination function. In these living bodies, even if foreign DNA has a long DNA sequence portion that is homologous to a specific region on the genome, it is incorporated into the specific region with low frequency, and it is incorporated at random into the genome in many cases.

Homologous recombination enables efficient modification of the existing genes. Since it can be used for the production of a new species of strains or the improvement of decreased functions of cells, a large number of attempts to increase the ratio of homologous recombination have been made in eukaryotic cells other than budding yeasts, to date.

For example, an attempt to construct a high expression system of the RAD51 gene, RAD52 gene, or the homolog gene thereof, which plays an important role in the homologous recombination of budding yeasts, has been made. However, it has been known that even if such RAD51 or RAD52 is allowed to express at a high level, homologous recombination ratio is increased only by approximately 2 or 3 times, and that it rather adversely affects cells (Yanez and Porter, 2002; Reiss et al., 2000). In addition, various types of targeting vectors have been developed to increase the ratio of homologous recombination. For example, a method to concentrate homologous recombinants (please refer to Patent Document 1 and Non-Patent Documents 1 and 2) based on the negative-positive selective method in mammalian cells or plant cells is a representative example. However, even if such a method is applied, homologous recombination frequency is still extremely low (1% or less). Moreover, since application of such a method requires complicated operations, this has not been a practical method.

With regard to studies about genetic recombination in eukaryotic cells other than budding yeasts, since genetic approach can easily be carried out, such studies have been conducted not only using fission yeasts but also using filamentous fungi. A type of filamentous fungi, *Neurospora crassa*, is one of organisms often used in studies regarding recombination. It has been known that the mei-3, mus-11, and mus-25 genes of *Neurospora crassa* are homologous to RAD51, RAD52, and RAD54, respectively, which function in the homologous recombination of budding yeasts. Thus, the ratio of homologous recombination of a mutant comprising a deletion

regarding these genes has been studied (Handa et al., 2000) by measurement of homologous-integration frequency of the mtr gene contained in the plasmid pMTR (Schroeder et al., 1995) into the chromosomal mtr locus as an indicator. Only 3% to 5% of transformants exhibited homologous integration in wild-type strain. In contrast, in the case of mei-3 and mus-25 mutants, almost no such homologous recombination took place. These data also showed that the ratio of homologous recombination is extremely low in *Neurospora crassa*, and that it is not easy to disrupt a specific gene by gene targeting.

On the other hand, it has been reported that a non-homologous recombination process progresses via DNA-dependent protein kinase (DNA-PKcs), a K70-Ku80 heterodimer, and a DNA ligase IV-Xrcc4 complex (please refer to Non-Patent Documents 3, 4, and 5). Thus, the inventor has conducted studies based on a working hypothesis that the ratio of homologous recombination would be increased by inhibition of the non-homologous mechanism.

Patent Document 1: Japanese Patent Application Laid-Open No. 2001-046053

Non-Patent Document 1: Terada et al., Nature biotech. 20, 1030-1034. 2002

Non-Patent Document 2: Jeannotte et al., EJ. Mol. Cell Biol. 11, 5578-5585. 1991

Non-Patent Document 3: Gallego et al., the Plant Journal, 35, 557-565. 2003

Non-Patent Document 4: Walker et al., Nature 412, 607-614. 2001

Non-Patent Document 5: Critchlow and Jackson, TIBS, 23, 394-398. 1998

DISCLOSURE OF THE INVENTION

Problems to be Solved by the Invention

Under the aforementioned circumstances, the present inventor has conducted intensive studies directed towards the discovery of a method of increasing the ratio of homologous recombination in eukaryotic cells. As a result, the inventor has unexpectedly found that the homologous recombination ratio in eukaryotic cells can

be increased by the loss of the functions of KU70 and KU80, which are genes necessary for non-homologous recombination, or by a decrease in such functions.

Accordingly, it is an object of the present invention to provide a method of increasing the ratio of homologous recombination.

In addition, it is another object of the present invention to provide high efficient homologous recombinant cells produced by the above method.

Means for Solving the problems

Thus, in order to increase the ratio of homologous recombination, the present invention provides a method of efficiently conducting homologous recombination, which comprises causing a decrease in the functions of genes that have been known to be necessary for non-homologous recombination or the loss of the functions thereof via means such as mutagenesis or gene disruption, and then introducing desired foreign DNA used to be conducted by homologous recombination into the genes.

The efficiency of homologous recombination realized using the method of the present invention is, for example, 70% or more, and more preferably 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more. Most preferably, 100% of homologous recombination frequency can be realized.

In general, cells used in the present invention may be either cells derived from tissues, or a cultured established cell line. Thus, the types of the cells used in the present invention are not limited, as long as they are eukaryotic cells. Suitable cells may include animal cells, plant cells, and fungal cells, which have a low ratio of homologous recombination.

Examples of animal cells used herein may include: mammalian cells such as those of a human, mouse, rat, bovine, swine, horse, chicken, sheep, feline, or canine; and those of Aves, reptiles, amphibians, and others.

Examples of plant cells used herein may include those of rice, soybean, wheat, barley, rye, cotton, starch, potato, peanut, and Arabidopsis.

Moreover, fungal cells wherein genetic manipulation is relatively easily carried out or other cells can also be used. The cells of filamentous fungi or the like are preferable. Examples of filamentous fungi used herein may include genus Neurospora, genus Aspergillus, genus Penicillium, genus Fusarium, genus Trichoderma, and genus Mucor. Of these, examples of preferred filamentous fungi, which are preferably used herein, may include: *Neurospora crassa*, *Neurospora sitophila*, *Neurospora tetrasperma*, and *Neurospora intermedia*, which belong to genus Neurospora; and *Aspergillus oryzae*, *Aspergillus sojae*, *Aspergillus niger*, *Aspergillus awamori*, *Aspergillus kawachi*, *Aspergillus parasiticus*, *Aspergillus flavus*, *Aspergillus nomius*, *Aspergillus fumigatus*, and *Aspergillus nidulans*, which belong to genus Aspergillus.

The types of genes necessary for non-homologous recombination used in the present invention are not limited, and those that have been known in the present technical field can be used. Preferred examples may include genes encoding DNA-dependent protein kinase (DNA-PKcs), a K70-Ku80 heterodimer, a DNA ligase IV-Xrcc4 complex, etc. In particular, the KU70 gene and/or the KU80 gene are most preferable.

Ku70 and Ku80 used in the present invention include the Ku70 and Ku80 homologs of any given eukaryotes. For example, genes including human Ku70 (P12956) (SEQ ID NO: 1), human Ku80 (P13010) (SEQ ID NO: 2), *Neurospora crassa* Ku70 (NCU08290.1) (SEQ ID NO: 3), *Neurospora crassa* Ku80 (NCU00077.1) (SEQ ID NO: 4), etc., and also genes, which encode an amino acid sequence comprising a deletion, addition, or substitution of one or several amino acids with respect to the gene products thereof, which have activity necessary for non-homologous recombination, can be used as the Ku70 and Ku80 homologs of the

present invention.

The type of a method of introducing DNA into cells used in the present invention is not particularly limited. Any method can be used, as long as it has been publicly known in the present technical field. Examples of such an introduction method used herein may include the spheroplast method, the electroshock method (electroporation method), the calcium phosphate method, and a method using cationic lipids. Of these, the electroshock method (electroporation method) is most preferable.

The present invention also provides cells having a significantly increased ratio of homologous recombination, which are produced by the method of the present invention.

Advantages of the Invention

Since the present invention achieves almost 100% of homologous recombination ratio in target cells, disruption, substitution, or the like of a gene of interest can efficiently be carried out. In addition, it becomes possible to insert the gene of a heterogeneous organism into the specific genome region of target cells, so as to allow the above gene to express therein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a method of substituting ncKU70 and ncKU80 with Hyg^r. A indicates a method of constructing an ncKU target vector. The 5'-region of the ncKU gene was amplified with (p-1) (SEQ ID NO: 5) and (p-2) (SEQ ID NO: 6), and the 3'-region thereof was amplified with (p-3) (SEQ ID NO: 7) and (p-4) (SEQ ID NO: 8). The Hyg^r gene was amplified with (p-5) (SEQ ID NO: 9) and (p-6) (SEQ ID NO: 10). B indicates homologous incorporation of a fusion PCR product into the target gene.

Figure 2 shows the comparison of the putative amino acid sequence of an

ncKu70 protein with the putative amino acid sequence of a human Ku70 protein. The frame with a solid line indicates a Ku70/80 DNA binding domain, the frame with a broken line indicates a Ku80 binding region, and the frame with a dotted line indicates an SAP domain. In addition, the region PHR particularly conserved in Ku of various types of organisms is indicated with an underlined italic type.

Figure 3 shows the comparison of the putative amino acid sequence of an ncKu80 protein with the putative amino acid sequence of a human Ku80 protein. The frame with a solid line indicates a Ku70/80 DNA binding domain, the frame with a broken line indicates a Ku80 binding region, and the frame with a dotted line indicates a DNA-PKcs binding domain. In addition, the region PHR particularly conserved in Ku of various types of organisms is indicated with an underlined italic type.

Figure 4 shows The UV and MMS sensitivity of a wild-type strain, the ncku70, and ncku80 strains. The open triangle indicates the wild-type strain, the filled circle indicates ncku70, and the filled square indicates ncku80. The experiment was carried out at least 3 times. Each point indicates the mean value of data.

BEST MODE FOR CARRYING OUT THE INVENTION

1. Identification of genes encoding factors necessary for non-homologous recombination in target cells

In the present invention, in order to introduce a function decrease or function loss into genes encoding factors necessary for non-homologous recombination, it is necessary to identify the genes encoding factors necessary for non-homologous recombination derived from the target cells. In the case of identifying a Ku70 or Ku80 homolog, for example, when the gene sequence of such a Ku70 or Ku80 homolog derived from the target cells has been unknown, the cDNA library, etc. of the target cells is screened based other known species such as the homolog gene sequence

of human Ku70 (SEQ ID NO: 1) or Ku80 (SEQ ID NO: 2). As a screening method, a method regarding nucleic acid hybridization and cloning, which has been publicly known in the technical field, is used, and the Ku70 or Ku80 homolog can be obtained by hybridization at a low, middle, or high stringent level. The "stringency" of the hybridization is easily determined by persons skilled in the art, and it is an empirical condition that depends on a probe length, a washing temperature, and a salt concentration. When hybridization screening is carried out to identify a homolog, persons skilled in the art can easily understand how to control a temperature, an ionic strength, or the like, while taking into consideration the length of a probe or the like.

Moreover, when the database of ORF derived from target cells exists, it is also possible to conduct BLAST search or the like against the above database, so as to identify a homolog of the known KU70 or KU80. In this case, it is also possible to clone KU70 or KU80 of interest by preparing suitable PCR primers used to amplify the corresponding gene as a whole based on the searched sequence, and then inserting the obtained PCR product into a suitable cloning vector.

The identified KU70 or KU80 is subcloned into a suitable cloning vector (for example, pUC19), so as to confirm its sequence.

2. Decrease or loss of functions of gene necessary for non-homologous recombination

The present invention provides a method of increasing homologous recombination frequency on the chromosome by causing a decrease in the functions of a gene necessary for non-homologous recombination existing in cells or the loss of the functions thereof. For such a purpose, the functions of a gene necessary for non-homologous recombination existing in cells can be modified. The type of such a method of modifying the functions is not limited. Examples of a method used herein may include methods publicly known to persons skilled in the art, such as a method of introducing a mutation into a gene necessary for non-homologous recombination

existing in cells, a method using RNA interference (RNAi), a method of disrupting the gene as a whole necessary for non-homologous recombination, or a method of introducing an antisense strand corresponding to a gene necessary for non-homologous recombination into cells. Preferred methods include a method of introducing a mutation into a gene necessary for non-homologous recombination existing in cells, a method using RNA interference (RNAi), and a method of disrupting the gene as a whole necessary for non-homologous recombination. More preferred methods include a method using RNA interference (RNAi) and a method of disrupting the gene as a whole necessary for non-homologous recombination. The most preferred method is a method of disrupting the gene as a whole necessary for non-homologous recombination.

As a method of disrupting the gene as a whole, there is a method of transforming cells with DNA, which has been produced by inserting a marker gene into the essential region of the cloned target gene. The DNA introduced into the cells induces homologous recombination via both sequences adjacent to the target gene, and it is able to disrupt the target gene on the chromosome via the marker gene (Alfa et al., 1993).

In addition, for the purpose of the loss of gene functions, RNA interference (RNAi) can be used. In this case, based on a nucleotide sequence associated with the function domain of a factor of interest that causes the function loss, short double-stranded RNA or a vector for generating the above RNA is introduced into cells, so as to bring on a decrease in the functions of the above factor or the loss of the functions thereof.

Moreover, as a method of introducing a mutation into cells *in vitro*, there are applied methods known in the present technical field, such as a site-directed mutagenesis or PCR mutagenesis. Such a site-directed mutagenesis (Carter, 1986; Zoller and Smith, 1987), a cassette mutagenesis, a target-selected mutagenesis (Wells

et al., 1985), or other known techniques, are carried out on DNA, which has been prepared for introduction of a mutation into a gene of interest and has been then cloned (Ausbel et al., 1987; Sambrook, 1989).

When a mutation is introduced into KU70 or KU80, so as to modify the functions thereof, it is desired to introduce a mutation such that the Ku70 protein or Ku80 protein activity can be lost, or to introduce a mutation into a site necessary for the interaction between the Ku70 protein and the Ku80 protein, so as to delete the above interaction.

3. Measurement of recombination activity

The degree of homologous recombination is measured, based on the ratio of the cells transformed by recombination at a homologous sequence site, to the cells transformed with DNA introduced from the outside of the cells.

Examples will be given below. However, these examples are not intended to limit the scope of the present invention.

Example 1

Neurospora crassa

1. Experimental materials

Table 1 shows the Neurospora strains used in the present experiment. C1-T10-37A and C1-T10-28a were used as wild types (Tamaru and Inoue, 1989). The *Escherichia coli* DH1 and XL-1 Blue strains were used to amplify plasmids (Sambrook et al., 1989).

The plasmids pBluescript SK^+ (Stratagene) and pGEM (Promega) were generally used to construct new vectors. The two plasmids pBARGEM7-1 (Pall and Brunelli, 1993) and pCSN43 (Staben et al., 1989), and the two cosmids G7H3 and G8B12 were acquired from Fungal Genetics Stock Center, University of ~~Kansas Medical School, Kansas City, KS 66160-7420~~ Missouri, Kansas City, 5007 Rockhill

Rd., Kansas City, MO 64110.

Table 1. *Neurospora crassa* strains used in the present invention

[Table 1]

Strain	Genotype	Source/Publication
C1-T10-37A	*A*	Stock in the laboratory
C1-T10-28a	*a*	Stock in the laboratory
54yo-728-5	*A ncku70*	Produced in the present experiment
54yo-728-7	*a ncku70*	Produced in the present experiment
54yo-828-3	*A ncku80*	Produced in the present experiment
54yo-828-4	*a nku80*	Produced in the present experiment
FGSC#2764	*A mei-3*	FGSC*
FGSC#6409	*A mus-11*	FGSC*

FGSC*: Fungal Genetics Stock Center

2. Methods

(1) Genetic research method of Neurospora

The gene analysis was carried out in accordance with the descriptions of Davis and de Serres (1970).

(2) PCR method

PCR amplification was carried out using Expand™ High-Fidelity PCR system (Roche Diagnostics Corp., Switzerland) in accordance with the instructions included therewith.

(3) Construction of plasmid used in substitution of KU homolog genes (hereinafter referred to as ncKU70 and ncKU80) of *Neurospora crassa* with Hyg gene

A method of substituting ncKU70 and ncKU80 with the

hygromycin-resistance gene Hyg^r is shown in Figures 1A and 1B.

(a) Preparation of DNA used in substitution of ncKU70

The 5'- and 3'-flanking DNAs of the Neurospora KU70 gene, each having a length of 2 kbp, were amplified by PCR using the cosmid G7H3 as a template (PCR conditions: after a reaction of 94°C and 2 minutes, a cycle consisting of 94°C and 15 seconds, 58°C and 30 seconds, and 72°C and 2 minutes, was repeated 10 times, and then, a cycle consisting of 94°C and 15 seconds, 58°C and 15 seconds, and 72°C and 2 minutes, was repeated 20 times (wherein the period of time for 72°C was extended by 5 seconds for every cycle), and thereafter, a reaction of 72°C and 7 minutes was further carried out, followed by conservation at 4°C).

5'-flanking DNA primers:

(p-1) 5'-GTGCTGTAGCCGTTTTGGGTATCGC-3' (SEQ ID NO: 5)

(p-2) 5'-GGCGTAATAGCGAAGAGATAGTTGCTGGAAATAA-3' (SEQ ID NO: 6)

3'-flanking DNA primers:

(p-3) 5'-AAGCATAAAGTGTAAAGGCTTGTTGATGACCGT-3' (SEQ ID NO: 7)

(p-4) 5'-TTGGACGCCGCACACCTCTCGCTCT-3' (SEQ ID NO: 8)

Subsequently, PCR amplification was carried out using the Hyg gene plasmid pCSN43 as a template (wherein PCR conditions were the same as those described above).

(p-5) 5'-TTATTTCCAGCAACTATCTCTTCGCTATTACGCC-3' (SEQ ID NO: 9)

(p-6) 5'-CACGGTCATCAACAAGCCTTTACACTTTATGCTT-3' (SEQ ID NO: 10)

The aforementioned three PCR products were mixed, and the obtained mixture was used as a fusion PCR template (Kuwayama et al., 2002). In addition, (p-1) (SEQ ID NO: 5) and (p-4) (SEQ ID NO: 8) were used as primers, so as to carry out fusion PCR under the following conditions: a cycle consisting of 94°C and 2 minutes, 94°C and 15 seconds, and 60°C and 30 seconds, was repeated 10 times, and then, a cycle consisting of 94°C and 15 seconds, 60°C and 30 seconds, and 68°C and 5

minutes, was repeated 20 times (wherein the period of time for 68°C was extended by 1 minute for every cycle), and thereafter, a reaction was carried out at 72°C for 7 minutes, followed by retention at 4°C).

The obtained fusion PCR product was electrophoresed on 0.7% agarose gel, and wild-type Neurospora was then transformed with it.

(b) Preparation of DNA used in substitution of ncKU80

The 5'- and 3'-flanking DNAs of the Neurospora KU80 gene, each having a length of 2 kbp, were amplified by PCR using the cosmid G8B12 as a template (wherein PCR conditions were the same as those for ncKU70).

5'-flanking DNA primers:

(p-7) 5'-GCGCCGGGAGGTTGTTCGTAAGCTG-3' (SEQ ID NO: 11)

(p-8) 5'-GGCGTAATAGCGAAGAGGCTTTTCGGCTTTGCTG-3' (SEQ ID NO: 12)

3'-flanking DNA primers:

(p-9) 5'-AAGCATAAAGTGTAAAGCAGGGTTGGAGACAGGT-3' (SEQ ID NO: 13)

(p-10) 5'-AAGGCGGAGTTGTTGGCTGCGAAGG-3' (SEQ ID NO: 14)

Subsequently, PCR amplification was carried out using the Hyg^r gene plasmid pCSN43 as a template (wherein PCR conditions were the same as those for ncKU70).

(p-11) 5'-CAGCAAAGCCGAAAAGCCTCTTCGCTATTACGCC-3' (SEQ ID NO: 15)

(p-12) 5'-ACCTGTCTCCAACCCTGCTTTACACTTTATGCTT-3' (SEQ ID NO: 16)

Fusion PCR was carried out using (p-7) (SEQ ID NO: 11) and (p-10) (SEQ ID NO: 14) under the aforementioned conditions.

(4) Electroshock method (electroporation method)

A conidiospore suspension was prepared at a concentration of 2.0×10^9 in 1 M sorbitol. 30 μl of the fusion PCR product was mixed with 50 μl of the conidiospore suspension, and the mixture was then incubated on ice for 5 minutes. Thereafter, 40 μl of the mixed solution was added to cells on an electroporator (BTX Electro Cell

Manipulation 600 Genetronics Inc.). The following conditions were applied to the electroshock: charged voltage: 1.5 kV; peak in the voltage/time mode: 2.5kV/resistance; time capacitance: 50 μF; and time resistance: R6 (186 ohms).

(5) Substitution of ncKU70 and ncKU80

After completion of the electroshock, 1 ml of Vogel's minimal medium that contained 1.2% sucrose was added to the resultant, and the obtained mixture was then incubated at 30°C for 2 hours. 200 μl of the obtained solution was applied to and expanded on an agar medium that contained hygromycin B (500 μg/ml). A hygromycin-resistance colony was isolated, and it was then confirmed by PCR whether or not substitution took place in the target locus. In addition, it was also confirmed by the Southern blot method whether or not it contained redundant Hyg^r gene copies.

(6) Mutagene sensitivity

Sensitivity to UV and methyl methanesulfonate (MMS) was examined according to the method described in the already issued publication (Inoue and Ishii, 1984).

3. Results

(1) Substitution experiment of ncKU70 and ncKU80

In order to discover a homolog of *Neurospora crassa* to human KU70 and KU80, search was conducted against Neurospora genome database (http://www-genome.wi.mit.edu/annotation/fungi/neurospora). The retrieved candidate genes were temporarily named as ncKU70 and ncKU80. The ncKU70 and ncKU80 genes encode 645 and 661 amino acids, respectively. Figure 2 shows the comparison of the amino acid sequence of human Ku70 with the amino acid sequence of ncKu70. Human Ku70 and ncKU70 had an identity of 23% and a similarity of 42%. Figure 3 shows the comparison of human Ku80 with ncKU80. Human Ku80 and ncKU80 had an identity of 23% and a similarity of 41%. G7H3 and G8B12

obtained from the Orbach/Sachs cosmid library (Orbach, 1994) contained ncKU70 and ncKU80, respectively. Accordingly, using these cosmids as templates, ncKU70 and ncKU80 were amplified by PCR. As described in the sections regarding materials and methods, a DNA fragment retaining a Hyg^r gene, to which 5'- and 3'-flanking DNAs with a length of 2 kbp derived from the ncku gene bound, was prepared by fusion PCR. The fusion PCR product was introduced into wild-type *Neurospora crassa*, and a hygromycin-resistance colony was then isolated. Approximately 200 transformants were subcloned, and genomic DNA was then extracted. Thereafter, it was confirmed by PCR whether or not the ncKU gene was substituted with the Hyg^r gene. As shown in Figure 1B, one of the PCR primers was designed outside of the ncKU gene, and the other PCR primer was designed inside of the Hyg^r gene. Approximately 10% of hygromycin-resistance colonies had the Hyg^r gene at the position of the ncKU gene. ncKU70 (the 54yo-728 strain retaining Hyg^r) and ncku80 (the 54yo-828 strain retaining Hyg^r) were used as ncku70 and ncku80 mutants, respectively. These strains were normal in terms of vegetative growth and homozygous cross proliferation. However, the strains were sensitive to UV to a small extent, and were clearly sensitive to MMS (Figure 4).

(2) Targeting of mtr gene and ad-3A gene in wild-type strain, ncku70, ncku80, mei-3, and mus-11 mutant strains

The mtr gene and ad-3A gene on the chromosomes IV and I were selected as targets of a substitution experiment. A mutant having a deletion in the mtr gene shows resistant to p-fluorophenylalanine (PFP), which is an amino acid analog. A mutant having a deletion in the ad-3A gene shows accumulation of purple pigments (please refer to the Neurospora Compendium, Academic Press, 2001). The mtr ORF or ad-3A ORF was substituted with the blasticidin-resistance gene bar, so as to construct a targeting vector. 2.7-kb DNA that contained the bar gene was cleaved from pBARG EM7-1 using restriction enzymes ScaI and SmaI.

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In the case of the mtr gene, construction and introduction of a targeting vector were carried out as follows. pMTR that contained the mtr gene was digested with MscI, and an approximately 1-kbp portion that contained an mtr gene promoter and a portion of ORF was eliminated. Subsequently, a 2.7-kbp bar fragment was inserted into the above portion, so as to produce the plasmid pGS1 (9.5 kbp). The plasmid pGS1 was digested with NotI. Thereafter, the thus obtained 6.7-kbp linear fragment that retained 1.8-kbp 5'- and 1.9-kbp 3'-flanking DNAs of the mtr gene at both ends of the bar gene was introduced into a strain having different genetic background by the electroporation method. Transformants that were resistant to blasticidin (200 μg/ml) were isolated, and it was then examined whether or not such transformants were resistant to PFP (20 μg/ml). If the bar-DNA had been changed for the mtr locus, it should have shown resistance to PFP. Thus, it was further examined whether or not recombination was carried out by homologous substitution. Table 2 shows that 10% to 30% of blasticidin-resistance transformants were generated as a result of homologous recombination in wild-type strains. In contrast, al the transformants of the ncku70 and ncku80 strains were generated as a result of homologous recombination. In the case of the mei-3 and mus-11 strains having a deletion in homologous recombination repair, almost no such homologous recombination took place.

[Table 2]

Strain	Experiment No.	Bla-resistance	PFP-resistance	Homologous recombination frequency (%)
Wild type	1	9	3	
	2	22	3	
	3	11	3	
	4	16	2	
	Total number	58	11	19
ncku70	1	41	41	
	2	18	18	
	Total number	59	59	100
ncku80	1	12	12	
	2	23	23	
	3	12	12	
	4	26	26	
	Total number	73	73	100
mei-3	1	58	2	
	2	35	1	
	Total number	93	3	3*
mus-11	1	45	0	
	2	20	0	
	Total number	65	0	0

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An ad-3A targeting vector that retained 1.6-kbp 5'- and 2-kbp 3'-flanking

DNAs of ad-3A at both sides was constructed in the same manner as in the case of the aforementioned mtr gene. The above vector was introduced into various types of strains. Blasticidin-resistance transformants were isolated, and sub-culture was then carried out in a minimal medium, to which adenine had been added, for 10 days. Thereafter, the number of colonies of purple mycelia, which indicate the loss of the functions of ad-3A, was counted. As shown in Table 3, approximately 50% of wild-type blasticidin-resistance transformants were purple mycelia. In contrast, 100% of the ncku70 and ncku80 transformants showed purple color. It was confirmed by PCR that these transformants were strains, which had been disrupted by homologous substitution (Table 3).

[Table 3]

Strain	Bla-resistance	Adenine requirement	Homologous recombination frequency (%)
Wild type	86	44	51
ncku70	46	46	100
ncku80	36	36	100

4. Relationship between targeting ratio and length of homologous sequence

In order to examine the relationship between the targeting ratio and the length of a homologous sequence, 50-, 100-, 500, and 1000-bps fragments, which were homologous to the 5'- and 3'-flanking DNAs of the mtr gene, were amplified by PCR, and they were then allowed to bind to both sides of the bar gene. It was examined whether or not the blasticidin-resistance transformant was resistant to PFP (Table 4). The blasticidin-resistance transformants, into which 50-bp and 100-bp homologous DNAs had been introduced, showed almost no PFP resistance in ncku70, ncku80, and

a wild-type strain. In the case of homology of 500-bp DNA, less than 10% of the blasticidin-resistance transformants showed PFP-resistance in the wild-type strain. In contrast, 90% or more of the blasticidin-resistance transformants showed PFP-resistance in the ncku70 and ncku80 strains. In the case of homology of 1000-bp DNA, less than 20% of the blasticidin-resistance transformants showed PFP-resistance in the wild-type strain. In contrast, all the blasticidin-resistance transformants showed PFP-resistance in the ncku70 and ncku80 strains. Accordingly, if the homologous portion has a length of at least 1000 bp, the target gene can be completely substituted.

[Table 4]

Strain	Length of homologous sequence	Experiment No.	Bla-resistance	PFP-resistance	Homologous recombination frequency (%)
Wild type	50 bp	1	22	0	
		2	29	1	
		Total number	51	1	2
ncku70	50 bp	1	30	0	
		2	29	1	
		Total number	59	1	2
ncku80	50 bp	1	29	0	
		2	30	0	
		Total number	59	0	0
Wild type	100 bp	1	20	0	
		2	30	1	
		Total number	50	1	2

ncku70	100 bp	1	8	2	
		2	13	0	
		Total number	21	2	10
ncku80	100 bp	1	14	0	
		2	32	2	
		Total number	46	2	4
Wild type	500 bp	1	40	4	
		2	40	3	
		Total number	80	7	9
ncku70	500 bp	1	40	35	
		2	39	37	
		Total number	79	72	91
ncku80	500 bp	1	30	28	
		2	39	36	
		Total number	69	64	93
Wild type	1,000 bp	1	9	3	
		2	22	7	
		3	40	6	
		4	40	7	
		Total number	111	23	21
ncku70	1,000 bp	1	4	4	
		2	8	8	
		3	10	10	
		4	8	8	
		Total number	30	30	100
ncku80	1,000 bp	1	31	31	
		2	19	19	

Total number	50	50	100

Example 2

Aspergillus

1. Cloning of KU70 gene of *Aspergillus nidulans*

Using BLAST network service, clone information having high homology to the KU70 gene of *Neurospora crassa* was searched against genome database of *Aspergillus nidulans* (http://www-genome.wi.mit.edu/annotation/fungi/aspergillus/index.html). As a result, it was considered that Contig 1.132 contained the full-length KU70 gene of *Aspergillus nidulans*. The putative Ku protein of *Aspergillus nidulans* showed homology of approximately 50% to the Ku70 protein of *Neurospora crassa*. Using the genomic DNA of the *A. nidulans* FGCS A89 strain as a template, a 4045-bp fragment that contained the full-length KU70 gene was amplified by PCR with the following primers.

KU70/For 5'-GAGAACTGATCATGCGATGCGTGGC-3' (SEQ ID NO: 17)

KU70/Rev 5'-CATTCGCTTGATCGACATGGTTGGC-3' (SEQ ID NO: 18)

Genomic DNA	1 (50 ng)
Primer Forward	1 (100 pmol)
Primer Reverse	1 (100 pmol)
10 x reaction buffer	5
dNTP mixture	4
Ex Taq	1
DDW (distilled water)	37
Total amount	50 μl

The above reaction system was prepared, and the PCR reaction was then carried out using TaKaRa PCR Thermal Cycler PERSONAL (Takara Shuzo Co., Ltd.).

The reaction conditions consisted of: 1 cycle of 96°C and 2 minutes; and 30 cycles of 96°C and 30 seconds, 58°C and 30 seconds, and 72°C and 7 minutes. After completion of the PCR reaction, the reaction product was subjected to agarose gel electrophoresis, so as to recover a fragment of interest from the gel. The obtained fragment was ligated to the pGEM-T Easy vector (Promega) by the TA cloning method, so as to obtain pGEMku70.

2. Disruption of KU70 gene of *Aspergillus nidulans* -oliC31-

Using pGEMku70 as a template, the HindIII site was introduced by the QuikChange site-directed mutagenesis method with the following primers. The underlined portions indicate HindIII sites.

KU70/(HindIII)/For 5'-CACATTGTCCAAGCTTACTGTGGCTACCC-3' (SEQ ID NO: 19)

KU70/(HindIII)/Rev 5'-GGGTAGCCACAGTAAGCTTGGACAATGTG-3' (SEQ ID NO: 20)

pGEMku70	2 (20 ng)
Primer Forward	2 (200 ng)
Primer Reverse	2 (200 ng)
10 x reaction buffer	5
dNTP mixture	4
Pfu Turbo	1
DDW (distilled water)	34
Total amount	50 μl

TaKaRa PCR Thermal Cycler PERSONAL was used for the PCR reaction. The reaction conditions consisted of: 1 cycle of 95°C and 2 minutes; and 18 cycles of 95°C and 30 seconds, 54°C and 30 seconds, and 70°C and 15 minutes. The obtained mutant plasmid was named as pGEMku70(H), and this plasmid was then digested with

BlnI and HindIII. Thereafter, a fragment obtained by digesting the *Aspergillus nidulans*-derived oligomycin-resistance gene oliC31 with BlnI and HindIII was inserted therein, so as to obtain pGEMku70::oliC31. This plasmid was digested with ApaI, so as to convert it to a linear form, and it was then introduced into the *A. nidulans* FGSC A89 strain by the protoplast PEG method. Transformants were selected in a potato dextrose medium that contained oligomycin (3 μg/ml). The obtained transformants were subjected to PCR and the Southern blot analysis, so as to select KU70 gene-disrupted strains.

3. Disruption of KU70 gene of *Aspergillus nidulans* -ptrA-

Using pGEMku70 as a template, the HindIII site was introduced by the QuikChange site-directed mutagenesis method with the following primers. The underlined portions indicate HindIII sites.

KU70/(HindIII)/For 5'-CACATTGTCCAAGCTTACTGTGGCTACCC-3' (SEQ ID NO: 21)

KU70/(HindIII)/Rev 5'-GGGTAGCCACAGTAAGCTTGGACAATGTG-3' (SEQ ID NO: 22)

pGEMku70	2 (20 ng)
Primer Forward	2 (200 ng)
Primer Reverse	2 (200 ng)
10 x reaction buffer	5
dNTP mixture	4
Pfu Turbo	1
DDW (distilled water)	34
Total amount	50 μl

TaKaRa PCR Thermal Cycler PERSONAL was used for the PCR reaction. The reaction conditions consisted of: 1 cycle of 95°C and 2 minutes; and 18 cycles of 95°C and 30 seconds, 54°C and 30 seconds, and 70°C and 15 minutes. The obtained

mutant plasmid was named as pGEMku70(H), and this plasmid was then digested with BlnI and HindIII. Thereafter, a fragment obtained by digesting the *Aspergillus oryzae*-derived pyrithiamin-resistance gene ptrA with BlnI and HindIII was inserted therein, so as to obtain pGEMku70:: ptrA. This plasmid was digested with SpeI, so as to convert it to a linear form, and it was then introduced into the *A. nidulans* FGSC A89 strain by the protoplast PEG method. Transformants were selected in a Czapek-Dox medium that contained pyrithiamin (100 μg/ml). The obtained transformants were subjected to PCR and the Southern blot analysis, so as to select KU70 gene-disrupted strains.

4. Disruption efficiency of any given gene –kexB-

In order to examine the gene disruption efficiency using a wild-type strain or ku70-disrupted strain as a host, the efficiency was obtained by kexB gene disruption. It was clear that the kexB gene encodes processing protease KexB, and that the kexB gene-disrupted strain forms a more compact colony than a wild-type strain does. Thus, a kexB gene disruption plasmid was introduced into a wild-type stain and a ku70-disrupted strain, and the ratio of the obtained transformants that showed the phenotype of a kexB gene disrupted strain was then examined. The results are shown below.

kexB gene disruption efficiency

Wild-type strain (FGSC A89) hosts 6/83 transformants (7.2%)

ku70 gene disrupted stain hosts 90/100 transformants (90%)

Hence, it was succeeded that the kexB gene was disrupted at an extremely high efficiency, and it was shown that the ku70 gene-disrupted strain is a strain significantly useful for genetic analysis such as gene disruption.

Example 3

Arabidopsis

1. Experimental materials

Table 5 shows the Arabidopsis strains used in the present experiment.

Any type of target gene may be used. In this experiment, AG (At4G18960) and LFY (At5g61850) were used as target genes. As a transformation marker, the GFP gene derived from the plasmid CaMV35S-sGFP(S65T)-NOS3' was used. The GFP gene was cut out of the plasmid using HindIII and EcoRI. Thereafter, it was blunt-ended and was then inserted into the EcoRV site of pBluescript SK^+ (pSKGFP). An approximately 2-kb portion located upstream of the read codon of the AG gene was amplified with a primer, to which a restriction site had been added, and it was then inserted into the site upstream of GFP of pSKGFP. Likewise, an approximately 2-bp portion located downstream of the read codon of the AG gene was inserted into the site downstream of GFP. The thus obtained portion ranging from pSKAG::GFP to AG::GFP was cut out, and it was then used for transformation. The same above operations were performed also on the LFY gene.

[Table 5]

Disrupted gene	GenBank registration No.	Name of stock strain	Nottingham stock No.	Homolog name
At1g16970	BH750130	SALK 037071	N537071	K70-1
	BZ378077	SALK106654	N606654	K70-2
	BZ292117	SALK 123114	N623114	K70-6
	BH750124	SALK 037064	N537064	K70-3
At1g48050	BH814153	SALK 065823	N565823	K80-1
	BZ762137	SALK 089730	N589730	K80-2

	BH814138	SALK 065799	N565799	K80-4
	BH254483	SALK 016627	N516627	K80-3
At5g57160	BH864398	SALK 095962	N595962	L4-1
	BH754746	SALK 044027	N544027	L4-2

2. Confirmation of gene homo-disrupted strain

(1) The seeds of each strain were sterilized with 5% hypochlorous acid, and they were then inoculated in an agar medium, to which Hyponex and sucrose had been added.

(2) 10 days later, a piece of cotyledon was cut out, and DNA was then separated using Microsmash manufactured by TOMY SEIKO Co., Ltd. Using the DNA as a template, PCR was carried out with primers suitable for genomic DNA consisting of 500 bases before and after a T-DNA insertion site. Individuals, wherein amplification of genomic DNA had not been observed, were temporarily defined as gene disruption homo individuals, and they were then used in the subsequent experiment.

3. Maintenance of KU and Lig4 disrupted strain

Individuals, which had been confirmed to be homo-disrupted strains, were cultivated according to common methods, so as to maintain the strains.

4. Establishment of cell culture system and transformation

(1) Hypocotyls and leaves were cut out of young plants (which were sterilized plants allowed to grow by the aforementioned method), 2 weeks after the inoculation, and they were then sectioned at a width of approximately 1 mm.

(2) The obtained sections were placed in a callus induction medium (CIM medium produced by adding MES, sugar, a fixation agent, and plant hormone, to a B5 medium).

(3) After the callus had sufficiently grown, it was transferred to a liquid medium (MS medium produced by adding sugar and plant hormone to an MS basal medium), and it was then subjected to a shake culture.

(4) It was replanted every 1 week.

(5) A small callus was directly used in transformation (when the size of a callus was great, it was converted to a protoplast, and the means described in 5. later was applied).

(6) Calluses were collected by centrifugation, and they were then suspended in an EP buffer (70 mM KCL, 0.3 M mannitol, 5 mM 2-morpholinoethanesulfonic acid, pH 5.8). The concentration of the suspension was controlled to 1.5 x 10^6/ml. 800 μl of the suspension was placed in an electroporation cuvette (4 mm gap). Using ECM 600 manufactured by BTX, electroporation was carried out under conditions consisting of mode: LV; capacitance: 125 μF; resistance: 0; charged voltage: 300 volts; field strength: 750 V/cm; and pulse length: 22-26 msec. 10 μg of DNA was used in a single electroporation. (7) After completion of the electroporation, the resultant was suspended in 5 ml of a liquid medium, to which 0.5 M mannitol had been added, and the mixture was left at rest for 1 hour.

(8) Agar was added to the liquid medium, and the obtained mixture was inoculated in a consolidated Petri dish medium.

5. Production of protoplast and transformation

(1) Hypocotyls and leaves were cut out of young plants (which were sterilized plants allowed to grow by the aforementioned method), 2 weeks after the inoculation, and they were then sectioned at a width of approximately 1 mm.

(2) The obtained sections were immersed in a 0.5 M mannitol solution for 1 hour, and they were then treated with an enzyme solution (1% cellulose Onozuka RS, 0.25% macerozyme R-10, 0.5 M mannitol, 8 mM calcium chloride, pH 5.5) for 5 to 10 hours. Thereafter, they were washed with 0.5 M mannitol 3 times.

(3) The number of cells was controlled to 2 x 10^6/ml, and 400 μl of the solution was placed in an electroporation cuvette (2 mm gap). DNA to be introduced was added thereto, resulting in a concentration of 5 to 10 μg/ml. Using ECM 600 manufactured

by BTX, electroporation was carried out under conditions consisting of mode: LV; capacitance: 500 μF; resistance: R3 (48 ohms); charged voltage: 106 volts; field strength: 530 V/cm; and pulse length: 22-26 msec.

(4) After completion of the electroporation, the resultant was suspended in 3 ml of a modified 8p medium, and the mixture was left at rest for 1 hour.

(5) The modified 8p medium was expanded on a medium consolidated with agarose, followed by culture.

6. Confirmation of transformant

(1) Ultraviolet ray was applied to callus that had grown after the transformation experiment.

(2) Each cell mass, wherein GFP expression had been confirmed, was replanted.

(3) After the cell mass had grown to a size of 5 mm, it was divided into two portions. One portion was transferred to a redifferentiation medium (RIM produced by adding MES, sugar, and plant hormone, to a B5 medium), and DNA was separated from the other portion. The DNA separation method was the same as that in Example 1. Using the obtained DNA as a template, PCR was carried out with primers that had been designed in the GFP gene and at a target gene site. The cell mass, wherein a fragment of a predicted size had appeared, was defined as that whose gene had been disrupted.

7. Transformation using Agrobacterium

(1) The Ti vector pBI221H was treated with restriction enzymes, and a 35S promoter/GUS structural gene/NOS terminator portion was cut out.

(2) The aforementioned transformation DNA was inserted into the cut portion, and it was then introduced into Agrobacterium by electroporation. Agrobacterium, which was resistant to ampicillin and had a plasmid of interest, was selected.

(3) Agrobacterium having the transformation DNA was cultured at 28°C overnight, and it was then suspended in an immersion suspension medium, resulting in OD600 of

0.8.

(4) In the case of using a plant body, a pot was turned bottom up, and the flower bud portion was immersed therein for 15 minutes. Thereafter, the plant was cultured for about 1 month, and seeds were then collected. Thereafter, the collected seeds were allowed to be germinated, and those, wherein light emission due to GFP had been observed, were subjected to PCR.

(5) In the case of using callus, it was immersed in a cell suspension for 2 or 3 minutes, and it was then cultured in a coexisting culture medium (N6CO medium) for 3 days. Thereafter, the culture was sterilized with carbenicillin, and the presence or absence of GFP light emission was then examined. Only the light emission portion was cultured in an MS medium containing plant hormone for 1 month. Thereafter, light emission was confirmed again, and DNA was then separated, followed by confirmation by PCR.

8. Other transformation experiments

The linearized transformation DNA was also used in transformation with a particle gun and in cell fusion transformation using a protoplast.

Example 4

Concerning disruption of human KU70 gene

1. Obtainment of human KU70 gene information

Information regarding the nucleotide sequence of a human KU70 (G22P1) gene, exon, intron, or the like, was obtained from human genome database of Ensembl project (http://www.ensembl.org/) (http://www.ensembl.org/Homo_sapiens/geneview?gene=ENSG00000100419).

2. Determination of repetitive sequence position

The obtained nucleotide sequence information was sent to REPEATMASKER WEB SERVER (http://ftp.genome.washington.edu/cgi-bin/RepeatMasker), so as to search for a repetitive sequence, thereby determining the position of such a repetitive sequence existing in the KU70 gene.

3. Design of primers used for targeting vector

Based on the nucleotide sequence information obtained in 1 and 2 above, PCR primers used to produce homology arms, which were used for targeting vectors, were designed. Primer sequences are shown below (underlined portions indicate restriction sites used in subcloning).

(1) Case of construction of poly A selection method vector

Primer pair used for 5'-side homology arm

Ku70 P2 AscI

5'-GCTATAGGCGCGCCTCTGCATTTAAGGAGAGAATAGCTGTG-3' (SEQ ID NO: 23)

Ku70 P3 NotI

5'-AGAACAGCGGCCGCAAGAGATCTCGATCACTGCTTATGATC-3' (SEQ ID NO: 24)

Primer pair used for 3'-side homology arm

Ku70 P4 NotI 5'-CTTTAGGCGGCCGCAATTCAAGATGAGTCATAAGAGGATC-3' (SEQ ID NO: 25)

Ku70 P5 SalI 5'-CATGTCGACATTTCAAGACAGGTGAAGAGGTGACAAG-3' (SEQ ID NO: 26)

(2) Case of construction of promoterless method vector

Primer pair used for 5'-side homology arm

Ku70 P2 SalI 5'-GCTATAGTCGACTCTGCATTTAAGGAGAGAATAGCTGTG-3' (SEQ ID NO: 27)

Ku70 P3 NotI

5'-AGAACAGCGGCCGCAAGAGATCTCGATCACTGCTTATGATC-3' (SEQ ID NO: 28)

Primer pair used for 3'-side homology arm

Ku70 P4 NotI 5'-CTTTAGGCGGCCGCAATTCAAGATGAGTCATAAGAGGATC-3'

(SEQ ID NO: 29)

Ku 70 P5 SalI

5'-ATCGCAGGCGCGCCAGACAGGTGAAGAGGTGACAAGATAC-3' (SEQ ID NO: 30)

4. Synthesis of homology arm by PCR method

(1) Materials and devices used

Human colon cancer-derived HCT116 genomic DNA (500 ng/μl)

KOD-Plus (thermal tolerance DNA polymerase manufactured by Toyobo)

PCR thermal cycler (TaKaRa Thermal Cycler MP, Model No. TP-3000, manufactured by Takara Shuzo Co., Ltd.)

Primer Mix, wherein each primer pair was prepared to a concentration of 10 pmol (please refer to 3 above)

Composition of PCR reaction solution:

HCT116 genomic DNA (500 ng/μl)	1 μl
10 x PCR buffer for KOD-Plus	5 μl
2 mM dNTPs	5 μl
25 mM MgSO4	2 μl
Primer mix (10 pmol each)	1.5 μl
DMSO	2.5 μl
KOD-Plus-DNA polymerase	1 μl
Sterilized water	32 μl
Total amount	50 μl

A PCR reaction was carried out with the aforementioned reaction system, using a PCR cycle cycler.

The reaction conditions consisted of: 1 cycle of 94°C and 2 minutes; and 30

cycles of 94°C and 15 seconds, 60°C and 30 seconds, and 68°C and 5 minutes.

After completion of the reaction, a DNA fragment as a PCR product was purified, and it was then digested with restriction enzymes at restriction sites added to each primer.

(2) Case of construction of poly A selection method vector

The PCR product DNA fragment of the 5'-side homology arm primer pair (Ku70 P2 AscI/Ku 70 P3 NotI) was digested with both reaction enzymes, AscI and NotI. This DNA fragment was named as Ku70 F2.

The PCR product DNA fragment of the 3'-side homology arm primer pair (Ku70 P4 NotI/Ku 70 P5 SalI) was digested with both reaction enzymes, SalI and NotI. This DNA fragment was named as Ku70 F3.

(3) Case of construction of promoterless method vector

The PCR product DNA fragment of the 5'-side homology arm primer pair (Ku70 P2 SalI/Ku 70 P3 NotI) was digested with both reaction enzymes, SalI and NotI. This DNA fragment was named as Ku70 F2B.

The PCR product DNA fragment of the 3'-side homology arm primer pair (Ku70 P4 NotI/Ku 70 P5 AscI) was digested with both reaction enzymes, NotI and AscI. This DNA fragment was named as Ku70 F3B.

Each DNA fragment was subcloned into a pBC subcloning vector, and both ends of the DNA fragment were sequenced, so as to confirm that it was a DNA fragment containing the KU70 gene.

5. Construction of targeting vector plasmid

(1) Case of construction of poly A selection method vector

The DNA fragment was inserted into cloning site-modified pMC1DT-3 in the order of Ku70 F2, a puromycin-resistance gene with a promoter, and Ku70 F3, thereby constructing a poly A selection vector. The plasmid was named as pPAS-Ku70 Puro.

(2) Case of construction of promoterless method vector plasmid

The DNA fragment was inserted into cloning site-modified pMC1DT-ApA in the order of Ku70 F2B, a poly A signal-added neomycin-resistance gene without a promoter, and Ku70 F3B, thereby constructing a poly A selection vector. The plasmid was named as pBDTA-Ku70 neo.

Both targeting vector plasmids were digested with restriction enzyme AscI, so as to linearize plasmid DNA.

6. Selection of KU70 gene-disrupted cells

(1) Gene disruption with poly A selection method vector

The linearized pPAS-Ku70 Puro DNA produced in 5 above was introduced into HCT116 cells by the electroporation method. In order to select cells wherein the KU70 gene had been disrupted by homologous recombination, culture was carried out in a puromycin-added McCoy 5A medium (final concentration of puromycin: 0.3 μg/ml), so as to form colonies. The obtained transformed cell colonies were picked up, and cells, wherein either one of two KU70 loci had been disrupted, were selected by the PCR method and the Southern blot analysis.

(2) Gene disruption with promoterless method vector plasmid

Subsequently, in order to disrupted either one locus, the linearized pBDTA-Ku70 neo was introduced into the cells by the same above method, and culture was then carried out in a G418-added McCoy 5A medium (final concentration of G418: 300 μg/ml), so as to form colonies. The obtained transformed cells were analyzed by the same method as that in (1) above, so as to select cell strains, wherein the second locus had also been disrupted.

(7) Disruption efficiency of any given gene –HPRT-

In order to examine gene disruption efficiency using human cell line HCT116 or the above human cell line-derived Ku70-disrupted cells, HPRT (hypoxanthine guanine phosphoribosyl transferase) gene disruption was carried out to obtain the above efficiency.

Since HPRT is an enzyme that functions in the salvage pathway of nucleic acid precursor synthesis, even if the HPRT gene is disrupted, it does not affect cell growth. Thus, gene disruption efficiency can accurately be measured using such HPRT. Plasmid DNA used in HPRT gene disruption, which had a hygromycin-resistance gene, was introduced into HCT116 cells and into the HCT116-derived ku70 disrupted cells. Thereafter, the ratio of the obtained hygromycin-resistance clones, wherein the HPRT gene had been disrupted, was examined by the PCR method and the Southern blotting method.

HPRT gene disruption efficiency (the number of gene-disrupted cells/the number of hygromycin-resistance cells)

Parent strain HCT116: 6/120 (5%)

ku70-disrupted cell strain: 30/120 (25%)

Thus, since the HPRT gene could be disrupted at an extremely high efficiency, it was shown that the ku70 gene-disrupted cell strain is a cell strain that is extremely useful for genetic analysis such as gene disruption.

INDUSTRIAL APPLICABILITY

According to the present invention, freely designed DNA can be incorporated into the specific site of the genome of desired cells with high probability. Thus, the present invention provides a gene recombination technique with high accuracy. In addition, since it becomes possible to develop an excision system using Cre/lox, for example, towards unnecessary incorporation possibly occurring during recombination, such unnecessary incorporation can also be eliminated. As a result, a specific gene can reliably be introduced into desired cells, and the gene can stably be expressed therein. Moreover, it is also possible to disrupt a specific gene with reliability. Accordingly, by applying the method of the present invention, a new variety can easily be improved in the field of producing food or the like using microorganisms.

Furthermore, according to the present invention, genes giving disadvantages to industrially applicable living bodies, such as a gene inhibiting matter production or a gene associated with a cytotoxic production system, can easily be eliminated.

Still further, by applying the method of the present invention to higher animals and plants, not only the breeding of the animals and plants, but also the development of a novel technique in the drug discovery and gene therapy in medical field, is anticipated.

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